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Treatment of Experimental Anthrax with Recombinant Capsule Depolymerase[▽]

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Bacillus anthracis **produces an antiphagocytic gamma-linked poly-D-glutamic acid capsule that is required for virulence. Capsule depolymerase (CapD) is a membrane-associated poly-γ-glutamate-specific depolymerase encoded on the *B. anthracis* capsule plasmid, pX02, that is reported to contribute to virulence by anchoring the capsule to the peptidoglycan and partially degrading high-molecular-weight capsule from the bacterial surface. We previously demonstrated that treatment with CapD effectively removes the capsule from anthrax bacilli, rendering them susceptible to phagocytic killing in vitro. Here we report that CapD promoted in vivo phagocytic killing of *B. anthracis* bacilli by mouse peritoneal neutrophils and that parenteral administration of CapD protected mice in two models of anthrax infection. CapD conferred significant protection compared with controls when coinjected with encapsulated bacilli from fully virulent *B. anthracis* Ames or the nontoxigenic encapsulated strain ΔAmes and when injected 10 min after infection with encapsulated bacilli from *B. anthracis* Ames. Protection was also observed when CapD was administered 30 h after infection with *B. anthracis* ΔAmes spores, while significant protection could not be demonstrated following challenge with *B. anthracis* Ames spores. These data support the proposed role of capsule in *B. anthracis* virulence and suggest that strategies to target anthrax bacilli for neutrophil killing may lead to novel postexposure therapies.**

Bacillus anthracis, the causative agent of anthrax, produces a plasmid-encoded antiphagocytic poly-γ-D-glutamic acid capsule that surrounds the vegetative bacillus form of the bacterium (16). *B. anthracis* also produces AB-type toxins consisting of protective antigen (PA), lethal factor (LF), and edema factor (EF) that contribute to virulence. LF is a zinc-dependent metalloprotease that cleaves and inactivates components of the mitogen-activated protein kinase signal transduction pathway (11), while EF is an adenylate cyclase that increases intracellular concentrations of cyclic AMP (25).

Neutrophils are a primary component of the innate immune system and vital for eliminating bacterial pathogens from the blood and tissues. During phagocytosis, neutrophils release intracellular granule components into the phagolysosome that contain reactive oxygen intermediates, antibacterial enzymes, and antimicrobial peptides that contribute to rapid killing of ingested bacteria. While neutrophils are reported to have a variable role in resistance to anthrax spore infection in mice (7), mouse neutrophils have been shown to kill unencapsulated *B. anthracis* (42). It has recently been demonstrated that human neutrophils kill encapsulated *B. anthracis*, although the killing was modest and predominantly extracellular (28), and we have previously reported that they can reduce viability of decapsulated bacilli by up to 3 logs while having minimal activity against encapsulated bacilli (39). The *B. anthracis* capsule (pX02) and toxin (pX01) plasmids encode factors that enhance survival in the host, likely in part by subverting the bactericidal activity of neutrophils. The capsule is essential for virulence (3, 10, 18, 21), and its antiphagocytic property is a primary mechanism of immune cell evasion utilized by *B. anthracis* (21, 22, 26). The anthrax lethal toxin (PA plus LF) and edema toxin (PA plus EF) are known to inhibit some neutrophil functions (1, 8, 12, 21, 34, 43), but in contrast to a deleterious effect of lethal toxin on macrophage survival from some animals (13, 14), neither affects human neutrophil viability (8). Methods to counter the antihost properties of the capsule and toxins represent current areas of anthrax research and may lead to new vaccines and treatments.

Recent reports have demonstrated that anticapsule antibodies can opsonize *B. anthracis* (6, 37, 40) and that active and passive immunization protect mice against experimental anthrax (6, 19, 23, 24), suggesting that the capsule is an attractive vaccine and therapeutic target. The strategy of enzymatically removing capsule from the bacterial surface as an approach to treat infections dates back to the work of Avery and Dubos in 1931 (2). They demonstrated that injecting an enzyme capable of degrading the pneumococcal capsular polysaccharide could protect mice from pneumococcal infection, presumably by targeting the bacteria to phagocytic cells. A similar approach using enzymes to degrade capsule showed a delay in time to death of animals infected with *Cryptococcus neoformans* (15), and the recent work of Mushtaq et al. demonstrated that a capsule-degrading endosialidase could be used to treat *Escherichia coli* infections in mice, again by targeting the organism for phagocytic killing (30, 31). Although the advent of antibiotics curtailed this approach to therapy, the emergence of antibiotic-resistant bacterial pathogens may trigger a renewed interest in such treatments.

CapD is a member of the γ-glutamyltranspeptidase family that autocatalytically forms a heterodimer consisting of 35-kDa

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14. ABSTRACT

Bacillus anthracis produces an antiphagocytic gamma-linked poly-D-glutamic acid capsule that is required for virulence. Capsule depolymerase (CapD) is a membrane-associated poly-gamma-glutamate-specific depolymerase encoded on the B. anthracis capsule plasmid, pX02, that is reported to contribute to virulence by anchoring the capsule to the peptidoglycan and partially degrading high molecular weight capsule from the bacterial surface. We previously demonstrated that treatment with CapD effectively removes the capsule from anthrax bacilli, rendering them susceptible to phagocytic killing in vitro. Here we report that CapD promoted in vivo phagocytic killing of B. anthracis bacilli by mouse peritoneal neutrophils and that parenteral administration of CapD protected mice using two models of anthrax infection. CapD conferred significant protection compared with controls when co-injected with encapsulated bacilli from fully virulent B. anthracis Ames or the nontoxigenic encapsulated strain, DeltaAmes and when injected 10 min after infection with encapsulated bacilli from B. anthracis Ames. Protection was also observed when CapD was administered 30 h after infection with B. anthracis DeltaAmes spores while significant protection could not be demonstrated following challenge with B. anthracis Ames spores. These data support the proposed role of capsule in B. anthracis virulence and suggest that strategies to target anthrax bacilli for neutrophil killing may lead to novel postexposure therapies.

15. SUBJECT TERMS

Bacillus anthracis, anthrax, capsule, capsule depolymerase, recombinant, efficacy, laboratory animals, mic

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and 15-kDa polypeptides (5). The enzyme is thought to contribute to the virulence of *B. anthracis* by releasing low-molecular-weight capsule from the surface of bacilli (27) and was recently reported to anchor polyglutamate to the peptidoglycan layer (5). In our previous work we demonstrated that CapD treatment of encapsulated anthrax bacilli efficiently degrades the capsule, removing it from the surface of the bacilli and thereby rendering the bacteria susceptible to phagocytic killing (39). In this study, using two mouse models of infection we show that administration of CapD protected mice from anthrax, likely by promoting phagocytic killing of bacilli in vivo.

MATERIALS AND METHODS

Bacterial strains and spore preparation. *B. anthracis* Ames ($pX01^+$ $pX02^+$) and ΔAmes ($pX01^-$ $pX02^+$) (United States Army Medical Research Institute of Infectious Diseases collection) were cultured in brain heart infusion (BHI) broth (Becton Dickinson and Co., Sparks, MD) at 37°C with 0.8% sodium bicarbonate and 5% carbon dioxide. *B. anthracis* spores were generated as previously described (6).

Recombinant CapD. The *capD* gene encoding amino acids 28 to 528 was expressed in pET15b (EMD Biosciences, San Diego, CA) as previously described (39). An additional construct of the *capD* gene encoding amino acids 43 to 528 was cloned into pTYB12 and expressed using the IMPACT protein purification system (New England BioLabs, Beverly, MA). For some animal experiments the gene for a *B. anthracis* membrane lipoprotein (BA3927) was cloned into pTYB12 and the protein was purified as for CapD. Recombinant proteins were expressed and purified according to the manufacturers' instructions and stored in phosphate-buffered saline, pH 7.4 (PBS). Endotoxin was removed from protein preparations with EndoTrap Red columns (Cambrex Corp., Walkersville, MD) or Detoxi-Gel endotoxin removal columns (Pierce Biotechnology, Inc., Rockford, IL). To evaluate the polyglutamate capsule-degrading activity of recombinant CapD proteins, purified *B. anthracis* capsule (500 μg/ml) prepared as previously described (6) was incubated for 1 h at 37°C in Dulbecco's modified Eagle's medium containing CapD amino acids 28 to 528 (CapD 28–528; 50 μg/ml) or CapD amino acids 43 to 528 (CapD 43–528; 50 μg/ml), and the degraded capsule products were analyzed on a 1% agarose gel and visualized with StainsAll (Sigma, St. Louis, MO) as described previously (39). In some experiments CapD was inactivated by incubation at 75°C for 30 min. This destroyed the enzymatic activity as assessed by degradation of capsule on agarose gels.

Human neutrophils. Human neutrophils were purified from unvaccinated volunteers by Ficoll-Hypaque density gradient centrifugation followed by dextran sedimentation and used to examine CapD-mediated neutrophil killing as previously described (39). Briefly, purified human neutrophils were resuspended in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (DF10), recombinant CapD (20 μg/ml), and 10% human AB serum as a source of complement. Encapsulated bacilli were generated by germinating heat-shocked *B. anthracis* Ames spores in BHI with 0.8% sodium bicarbonate and 5% carbon dioxide for 90 min, washed with PBS, and added to give a neutrophil-to-bacillus ratio of 50:1 (5×10^6 neutrophils/ml to 1×10^5 bacilli/ml). Duplicate tubes were incubated for 2 h at 37°C on an Eppendorf tube rotator. After incubation, bacterial viability was measured by serial dilution in water and plating on Luria-Bertani agar.

Animals. Mice were obtained from the National Cancer Institute, Fort Detrick (Frederick, MD). Female BALB/c mice (6 to 8 weeks old) were used for *B. anthracis* ΔAmes bacillus challenge experiments. All other experiments were performed with female Swiss Webster mice (6 to 8 weeks old). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (31a). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Bacillus challenge model. *B. anthracis* Ames and ΔAmes spores were used to inoculate BHI broth containing 0.8% bicarbonate and grown at 37°C in 5% CO_2 overnight with shaking. The cultures were then diluted 1:1,000 in fresh BHI (0.8% bicarbonate) and grown for an additional 6 h (37°C in 5% CO_2) to approximately 1×10^7 CFU/ml. Encapsulated bacilli were harvested by centrifugation, washed twice in PBS, and diluted in PBS. At this time, the bacilli were in chains of 5 to 10 (*B. anthracis* Ames) or 10 to 20 (*B. anthracis* ΔAmes). Mice were challenged intraperitoneally (i.p.) with bacilli mixed with CapD (20 μg/ml), PBS or heat-inactivated CapD, and normal BALB/c or Swiss Webster mouse serum (10%) in a 200-μl volume 6 h after they had received an i.p. injection of 1 ml of a 2% starch solution. To examine the effect of CapD when administered after bacillus infection, Swiss Webster mice were injected with 2% starch and challenged 6 h later with encapsulated *B. anthracis* Ames bacilli in PBS. Ten minutes after infection, mice were injected i.p. with 300 μl active or heat-inactivated CapD (13.4 μg/ml) containing 10% serum in PBS.

To examine the effect of CapD on bacterial viability after challenge, mice administered i.p. 1 ml of 2% starch were injected i.p. 6 h later with 1×10^7 CFU of encapsulated *B. anthracis* Ames bacilli containing 50 μg/ml active or heat-inactivated CapD and 10% mouse serum. Two hours after injection, animals were euthanized and their peritoneal cavities were washed with 5 ml PBS. Bacterial viability in the i.p. exudate was measured by serial dilution and plating. Phagocytosis of bacilli by i.p. neutrophils was visualized by phase and fluorescence microscopy. Briefly, bacilli and phagocytes from i.p. exudates were concentrated in Eppendorf tubes. Extracellular, encapsulated bacilli were reacted with a mouse anticapsule monoclonal antibody (1:1,000) (9) followed by incubation with Alexa Fluor 594 goat anti-mouse secondary immunoglobulin G (1:5,000; Invitrogen Corp., Carlsbad, CA). Phagocytes were then permeabilized with 0.2% Triton X-100 in PBS, and intracellular bacilli were stained with fluorescein-labeled mouse anticapsule monoclonal antibody (1:1,000) (9). Intracellular and extracellular bacilli were then stained with 4′,6-diamidino-2-phenylindole dihydrochloride (1:5,000) (Invitrogen).

Spore challenge model. *B. anthracis* Ames or ΔAmes spores were washed twice with water for injection and heat shocked at 65°C for 40 min. Swiss Webster mice were infected i.p. with 200 μl of *B. anthracis* spore suspension. Purified CapD (2 mg/ml) in 200 μl PBS was administered i.p. concurrently with spore challenge and/or i.p. and intravenously (i.v.) in 200 μl PBS by tail vein 30 h after challenge. Thus, each injection (i.p. or i.v.) contained 400 μg of CapD, and at 30 h mice received a total of 800 μg.

Statistics. The statistical significance of differences in survival rates and mean survival time of treated compared to control mice was determined by Fisher's exact test and the Kaplan-Meier log rank test, respectively, using SAS software (SAS Institute Inc., Cary, NC). Differences in recovery of viable bacilli from mice were determined by Student's *t* test for unequal variances.

RESULTS

Recombinant CapD. The open reading frame of *capD* excluding the signal sequence and encoding residues 28 to 528, originally cloned and expressed in pET15b with a histidine tag, resulted in low yields after purification. An additional amplicon encoding amino acids 43 to 528 was cloned into pTYB12 as a self-cleavable intein fusion protein in an attempt to increase solubility and recovery of the recombinant protein and purified using the IMPACT-CN system. The CapD 43–528 fusion protein yielded approximately 10-fold-higher expression levels of CapD than did the pET15b histidine tag fusion, presumably due to increased solubility during expression in *E. coli*. The two forms of CapD had similar enzyme activities when tested on purified capsule as determined by agarose gel analysis of capsule degradation following incubation with enzyme (data not shown). CapD 43–528 and CapD 28–528 were next compared for their abilities to enhance neutrophil killing of encapsulated *B. anthracis* Ames bacilli. Treatment of encapsulated bacilli with either CapD 28–528 or CapD 43–528 resulted in a striking enhancement of neutrophil killing with >99% loss of viability in 2 h. In two experiments there was an average of 0.45% and 0.37% survival after CapD 28–528 and CapD 43–528 treatment, respectively, compared to 100% survival for untreated bacilli (data not shown). The higher yield and similar enzyme activity of CapD 43–528 expressed in pTYB12 prompted us to use this construct rather than CapD 28–528 expressed in pET15b.

CapD treatment of mice infected with *B. anthracis* bacilli. To evaluate the effect of CapD treatment on infection with *B.*

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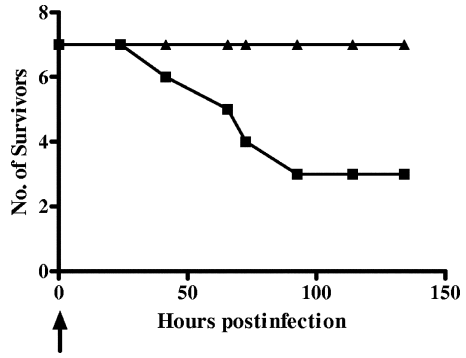

FIG. 1. CapD treatment of mice infected with *B. anthracis* ΔAmes bacilli. BALB/c mice were injected i.p. with 2% starch and challenged 6 h later with 1,000 CFU *B. anthracis* ΔAmes bacilli concurrently with either 4 μg CapD (▲) or PBS (■) as described in Materials and Methods and monitored for mortality. Survival rate and mean survival time were significantly greater in the CapD-treated than in the PBS group ($P = 0.035$, Fisher's exact test, and $P = 0.022$, log rank test, respectively). Administration of CapD is indicated by an arrow.

anthracis bacilli, we designed an experiment to expose CapD-treated bacilli to host phagocytes at the time of challenge to simulate the environment encountered by bacilli after spore germination and dissemination and to optimize the interaction with neutrophils and efficacy of treatment. BALB/c or Swiss Webster mice were administered i.p. 1 ml of a 2% starch solution which is known to stimulate the accumulation of neutrophils (42). Six hours later the mice were challenged i.p. with *B. anthracis* bacilli coinjected with CapD or PBS. BALB/c mice challenged with 1,000 CFU *B. anthracis* ΔAmes bacilli coinjected with CapD all survived (seven/seven), while only three of seven mice given PBS survived (Fig. 1, $P = 0.035$, Fisher's exact test). Mean survival time was also significantly increased by CapD treatment ($P = 0.022$, log rank test). No loss of bacterial viability was observed during incubation of bacilli in PBS containing 10% serum and CapD (data not shown), suggesting that bacilli treated with CapD were killed in vivo after infection.

To examine the effect of CapD treatment on a fully virulent strain, three experiments were performed. In the first experiment, seven Swiss Webster mice were challenged with 4,000 CFU of *B. anthracis* Ames bacilli coinjected with either active CapD or heat-inactivated CapD. All seven mice challenged with bacilli coinjected with heat-inactivated CapD succumbed within 18 h (Fig. 2A). Of the mice given active CapD coinjected with bacilli, all seven survived for at least 28 h, three succumbed by 42 h, and two succumbed by 66 h. The survival rate was not significantly increased by CapD treatment ($P > 0.1$, active versus heat-inactivated CapD, Fisher's exact test). However, mean survival time was significantly greater in the active than in the heat-inactivated CapD-treated group ($P = 0.0003$, log rank test). In the second experiment, eight mice per group were challenged with 500 CFU coinjected with active CapD or heat-inactivated CapD (Fig. 2B). An additional control group of mice that was not administered starch before challenge was included. This group was challenged with bacilli in PBS. All eight mice in this group as well as all eight mice given starch before challenge with bacilli coinjected with heat-inactivated CapD succumbed 18 to 22 h postinfection, similar

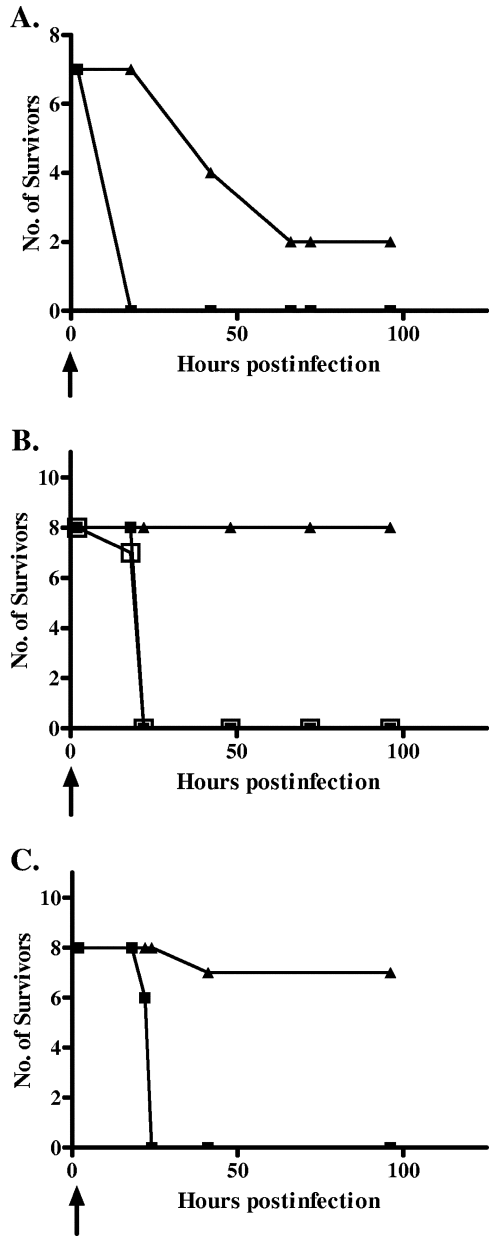

FIG. 2. CapD treatment of mice infected with *B. anthracis* Ames bacilli. Swiss Webster mice were injected i.p. with 2% starch and challenged 6 h later with 4,000 CFU (A) or 500 CFU (B) of *B. anthracis* Ames bacilli concurrently with 4 μg active CapD (▲) or heat-inactivated CapD (■) and monitored for mortality. In the experiment shown in panel C 4 μg active (▲) or heat-inactivated (■) CapD was injected 10 min after challenge with 200 CFU. In the experiment shown in panel B an additional control group not given starch was challenged with bacilli in PBS (□). In the experiment shown in panel A the survival rate was not significantly different after CapD treatment ($P > 0.1$, Fisher's exact test) while mean survival time was significantly greater in the CapD treatment group ($P = 0.0003$, log rank test). In panel B the CapD treatment resulted in a significantly increased survival rate ($P = 0.0005$, active versus heat-inactivated CapD, Fisher's exact test) and mean survival time ($P = 0.0003$, active versus heat-inactivated CapD, log rank test). All mice in the heat-inactivated CapD (■) and PBS (□) groups in panel B succumbed between 18 and 22 h postinfection. In panel C CapD treatment significantly increased survival ($P = 0.001$, active versus heat-inactivated CapD, Fisher's exact test) and mean survival time ($P = 0.0002$, active versus heat-inactivated CapD, log rank test). Administration of CapD is indicated by arrows.

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to results of the first experiment. By contrast, the eight mice administered bacilli coinjected with active CapD showed a significantly increased survival rate (Fig. 2B, $P = 0.0005$, Fisher's exact test) and mean survival time ($P = 0.0003$, log rank test). To examine the protective effect of CapD when given after infection, mice were challenged i.p. with 200 CFU of encapsulated *B. anthracis* Ames bacilli and injected i.p. 10 min later with active CapD or heat-inactivated CapD. All eight mice given heat-inactivated CapD died within 24 h, while seven of eight treated with active CapD survived, resulting in significantly increased survival rate and mean survival time (Fig. 2C, $P = 0.001$, Fisher's exact test, and $P = 0.0002$, log rank test, respectively). These data suggest that the protective effect of CapD was due to capsule removal by the active enzyme, which facilitated host killing and clearance of bacilli from the site of infection. Starch-elicited neutrophils by themselves did not protect mice against challenge with bacilli in the absence of active CapD.

To examine the effect of CapD treatment on bacillus survival in vivo, mice were injected i.p. with 2% starch and challenged i.p. 6 h later with 1×10^7 CFU of encapsulated bacilli containing active or heat-inactivated CapD. Two hours after challenge, mice were euthanized and the peritoneal exudates were collected and examined as described in Materials and Methods. The average percent recovery of viable bacilli in the exudate from mice injected with bacilli and heat-inactivated CapD was 84.9% ± 17.4% ($n = 4$). This is in contrast to the recovery of only 6.0% ± 2.6% ($n = 4$) in mice injected with bacilli and active CapD ($P = 0.0025$, Student's t test for unequal variances) (data not shown). On examination of the exudates under phase and fluorescence microscopy, numerous neutrophils from the exudates of mice that received CapD could be observed with internalized bacilli and most of the bacilli were intracellular. No capsule was observed by immunofluorescence on any of the bacilli. In contrast, after treatment with heat-inactivated CapD, only a rare neutrophil had ingested bacilli and most bacilli were extracellular and encapsulated (data not shown). Similar results were observed when the CapD was administered i.p. 10 min after the bacilli were injected.

CapD treatment of mice infected with *B. anthracis* spores. The protective effect of CapD when administered concurrently with spore challenge and postexposure was evaluated in Swiss Webster mice challenged with *B. anthracis* ΔAmes spores. Mice were challenged i.p. with 1,800 *B. anthracis* ΔAmes spores concurrently with 400 μg of CapD or PBS and subsequently given 400 μg of CapD or PBS both i.p. and i.v. at 30 h postinfection. Mice given CapD all survived (10/10) compared with 4 of 10 surviving in the PBS group (Fig. 3A, $P = 0.005$, Fisher's exact test). Analysis of the mean survival time also showed that CapD treatment was highly effective ($P = 0.004$, CapD versus PBS, log rank test).

To determine whether CapD could increase survival when administered postexposure but not concurrently with spore challenge, mice were administered 400 μg i.p. and i.v. of either CapD or an irrelevant control protein with no capsule-degrading activity 30 h after challenge with 6,600 *B. anthracis* ΔAmes spores. The control protein, a basic membrane lipoprotein, BA3927, was cloned into pTYB12 and purified by the same method as CapD was as described in Materials and Methods. All 10 mice receiving CapD survived, compared with five of nine in the group administered BA3927, resulting in a significantly enhanced survival rate and mean survival time (Fig. 3B, $P = 0.033$, Fisher's exact test; $P = 0.02$, log rank test, respectively), indicating that protection was CapD specific and that postexposure treatment with CapD was effective in increasing survival even when given 30 h after infection with *B. anthracis* ΔAmes spores.

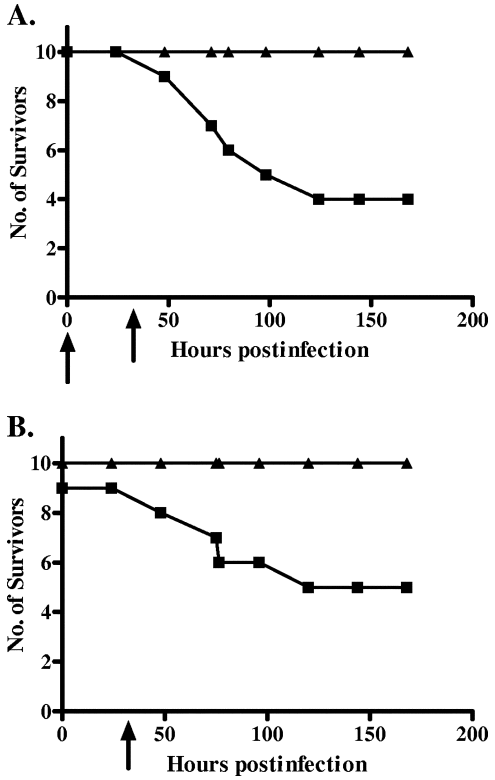

FIG. 3. CapD treatment of mice infected with *B. anthracis* ΔAmes spores. Swiss Webster mice in panel A were treated with CapD (▲) or PBS (■) at time zero (400 μg i.p.) and at 30 h (400 μg i.p. and i.v.) after challenge with 1,800 spores, and those in panel B were treated with CapD (▲) or BA3927 (■) only at 30 h (400 μg i.p. and i.v.) after challenge with 6,600 spores and monitored for mortality. In both experiments CapD treatment resulted in a significantly increased survival rate (panel A, $P = 0.005$, CapD versus PBS; panel B, $P = 0.033$, CapD versus BA3927, Fisher's exact test) and mean survival time (panel A, $P = 0.004$, CapD versus PBS; panel B, $P = 0.02$, CapD versus BA3927, log rank test). Administration of CapD is indicated by arrows.

Two experiments were next performed with the fully virulent *B. anthracis* Ames strain and using the spore challenge model. In both experiments, 400 μg active or heat-inactivated CapD was administered i.p. and i.v. 30 h postinfection using 10 mice per group. At 30 h after infection, several animals had already succumbed. In the first experiment, two mice in each group succumbed before treatment began, while one mouse in each group had succumbed in the second experiment. We did not observe statistically significant protection in either experiment when we examined survival rate ($P > 0.5$, Fisher's exact test) or mean survival time ($P > 0.1$, log rank test), although a higher rate of survival was observed in the CapD-treated group in both experiments (Fig. 4A and B).

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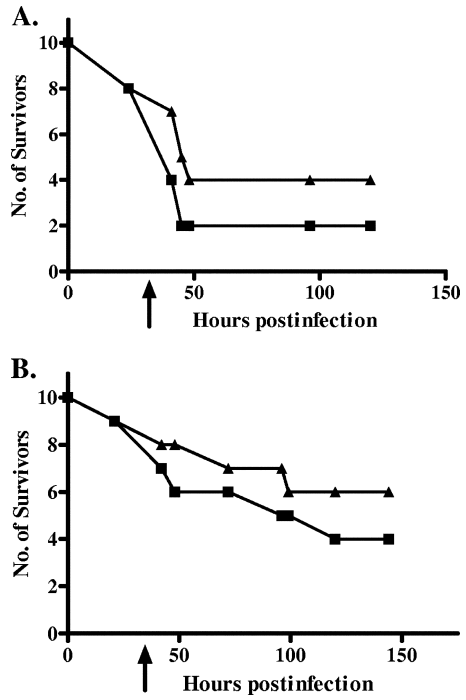

FIG. 4. CapD treatment of mice infected with *B. anthracis* Ames spores. Swiss Webster mice were treated with active CapD (▲) or heat-inactivated CapD (■) at 30 h (400 μg i.p. and i.v.) after challenge with 2,400 spores (A) or 560 spores (B). CapD treatment did not increase survival rate ($P > 0.5$, active versus heat-inactivated CapD, Fisher's exact test) or mean survival time ($P > 0.1$, active versus heat-inactivated CapD, log rank test) in either experiment. Administration of CapD is indicated by arrows.

DISCUSSION

The role of the capsule in virulence and promoting survival and replication in the host is well documented and thought to be related to its potent antiphagocytic property. Immune responses to the capsule protect animals from infection likely by inducing opsonizing antibodies, targeting encapsulated anthrax bacilli to phagocytes (6). We report here that treatment targeting *B. anthracis* bacilli to phagocytes by enzymatically removing the capsule can protect animals from infection, further supporting the role of capsule in evading host innate immunity. Although dormant spores may not be susceptible to phagocytic killing until they germinate (17, 20, 32), the vegetative form of the organism is susceptible to phagocytic killing, particularly when devoid of capsule (20, 28, 39, 42). As mentioned above, enzyme treatment to remove microbial capsules has been successfully used to treat existing infections with pneumococci, *Cryptococcus*, and *E. coli* (2, 15, 31) in mouse models of infection. In these studies, the authors attributed the observed protection to increased levels of phagocytosis resulting from capsule removal in vivo. It is possible that enzymatic removal of capsule exposes pathogen-associated molecular patterns on the bacterial surface, resulting in enhanced phagocytosis via activation of the alternative complement pathway. This is consistent with our previous observation that serum is necessary for neutrophil killing of CapD-treated encapsulated bacilli (39).

Two experimental methods were employed in our studies: i.p. administration of CapD concurrently with or 10 min after a bacillus challenge and postexposure i.p. and i.v. administration of CapD after a spore challenge. Significant CapD-mediated protection was observed for the *B. anthracis* ΔAmes and Ames bacillus challenge models as well as the *B. anthracis* ΔAmes spore challenge model. Statistically significant protection was not achieved when CapD was administered after *B. anthracis* Ames spore challenge, although the results were suggestive of protection. It should be emphasized that in these experiments CapD was given only at a single time point, and it is likely that the concentration of enzymatically active CapD may have decreased substantially due to inactivation by serum proteases as well as removal from the circulation. Indeed, preliminary experiments revealed a significant decrease in CapD activity after 5 h of incubation in mouse serum in vitro, and we could not detect activity in serum from a mouse bled 3 h after i.v. administration of 200 μg of CapD (data not shown). Thus, to optimize the use of CapD as a therapeutic, we are currently exploring ways to increase its stability and half-life in vivo.

Despite our inability to show protection in the Ames spore challenge model, we propose that enzymatic removal of capsule in vivo can sensitize anthrax bacilli to phagocytic killing and result in clearance of bacilli from the blood and infected organs. In these experiments we attempted to maximize exposure of encapsulated bacilli to CapD while in proximity to neutrophils and other phagocytes. We injected a 2% starch solution into the i.p. cavity of mice to stimulate influx of neutrophils before a bacillus challenge. Despite the presence of recruited neutrophils at the site of infection, mice receiving heat-inactivated CapD or PBS were susceptible to infection in the bacillus challenge model. Additionally, the nearly identical kinetics of infection and time to death after bacillus challenge of control starch-stimulated mice compared with control mice not injected with starch indicates that elicited peritoneal neutrophils by themselves do not protect against infection and that encapsulated bacilli were resistant to host phagocytic killing. In contrast, CapD treatment completely protected mice from bacillus challenge (Fig. 2B), demonstrating that enzymatic capsule removal facilitated efficient killing of *B. anthracis* bacilli in the host. CapD also conferred significant protection when given 10 min after infection (Fig. 2C), suggesting that it is enzymatically active and enhances neutrophil killing of bacilli in vivo. Our previous results showed that capsule is removed from bacilli after only a few minutes of treatment with CapD in vitro, after which they become susceptible to phagocytic killing (39). Furthermore, examination of the CapD-treated bacilli obtained from the peritoneal cavity 2 h after inoculation showed that CapD treatment resulted in removal of capsule from the bacillus and phagocytosis of the bacilli by neutrophils. After treatment with heat-inactivated CapD, bacilli remained encapsulated and extracellular. These results suggest that the mechanism of protection is related to the capsule-degrading activity of CapD, resulting in phagocytic killing of bacilli.

It is possible that other host factors such as serum complement or antimicrobial peptides may contribute to protection. However, we did not observe a decrease in bacterial viability during incubation of CapD-treated bacilli in mouse serum (data not shown). Some antimicrobial peptides or defensins

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are bactericidal for *B. anthracis* (29, 36, 41), and capsule does confer some resistance (33). Future studies will determine if CapD treatment sensitizes bacilli to antimicrobial peptides. Thus, the therapeutic effect of CapD could be mediated by neutrophil killing resulting from a combination of phagocytic and extracellular killing in which some bacilli were ingested and killed in phagolysosomes and some were killed by antimicrobial peptides found in neutrophil extracellular traps (4).

Enzymes generally have poor pharmacokinetic profiles, and only a few, such as streptokinase and plasminogen activator for thrombolytic blood clot therapy, have been approved for human use. The availability of antibacterial enzymes for the treatment of anthrax, however, may prove valuable, particularly against antibiotic-resistant strains for which no treatment may be available. Recent reports indicate that such an approach can be used to treat experimental infections with *B. anthracis* using phospholipase A_2 (35) or *Bacillus cereus* using phage lysin (38) when enzyme is administered shortly after infection. CapD, while not directly bactericidal, facilitates host cell phagocytic killing of encapsulated bacilli, possibly by exposing pathogen-associated molecular patterns and promoting complement deposition on the bacterial surface. In this respect, the use of enzymes to target bacteria for phagocytic killing is similar to passive immunization with anticapsule antibodies, an approach that has recently been reported to afford protection in animal models of anthrax (23, 24).

The threat of antibiotic- and/or vaccine-resistant *B. anthracis* emerging as a biological weapon increases the likelihood that alternative treatments for anthrax may be necessary. Capsule-degrading enzymes that remove the capsule that is essential for virulence and target *B. anthracis* bacilli to neutrophils and other components of innate immunity offer a novel approach that could be effective against such antibiotic- and/or vaccine-resistant strains.

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REFERENCES

1. **Abalakin, V. A., E. G. Sirina, and T. D. Cherkasova.** 1990. The effect of lethal anthrax toxin on the functional activity of peritoneal mononuclear phagocytes and polymorphonuclear neutrophils in mice. Zh. Mikrobiol. Epidemiol. Immunobiol. **2:**62–67.
2. **Avery, O. T., and R. Dubos.** 1931. The protective action of a specific enzyme against type III pneumococcus infection in mice. J. Exp. Med. **54:**73–89.
3. **Bail, O.** 1914. Changes to bacteria inside the animal body. IX. Regarding the correlation between capsule formation, spore formation and infectiousness of the anthrax bacillus. Zentralbl. Bakteriol. **75:**159–173.
4. **Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, and A. Zychlinsky.** 2004. Neutrophil extracellular traps kill bacteria. Science **303:**1532–1535.
5. **Candela, T., and A. Fouet.** 2005. *Bacillus anthracis* CapD, belonging to the gamma-glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. Mol. Microbiol. **57:**717–726.
6. **Chabot, D. J., A. Scorpio, S. A. Tobery, S. F. Little, S. L. Norris, and A. M. Friedlander.** 2004. Anthrax capsule vaccine protects against experimental infection. Vaccine **23:**43–47.
7. **Cote, C. K., N. Van Rooijen, and S. L. Welkos.** 2006. Roles of macrophages and neutrophils in the early host response to *Bacillus anthracis* spores in a mouse model of infection. Infect. Immun. **74:**469–480.
8. **Crawford, M. A., C. V. Aylott, R. W. Bourdeau, and G. M. Bokoch.** 2006. *Bacillus anthracis* toxins inhibit human neutrophil NADPH oxidase activity. J. Immunol. **176:**7557–7565.
9. **De, B. K., S. L. Bragg, G. N. Sanden, K. E. Wilson, L. A. Diem, C. K. Marston, A. R. Hoffmaster, G. A. Barnett, R. S. Weyant, T. G. Abshire, J. W. Ezzell, and T. Popovic.** 2002. A two-component direct fluorescent-antibody assay for rapid identification of *Bacillus anthracis*. Emerg. Infect. Dis. **8:**1060–1065.
10. **Drysdale, M., S. Heninger, J. Hutt, Y. Chen, C. R. Lyons, and T. M. Koehler.** 2005. Capsule synthesis by *Bacillus anthracis* is required for dissemination in murine inhalation anthrax. EMBO J. **24:**221–227.
11. **Duesbery, N. S., C. P. Webb, S. H. Leppla, V. M. Gordon, K. R. Klimpel, T. D. Copeland, N. G. Ahn, M. K. Oskarsson, K. Fukasawa, K. D. Paull, and G. F. Vande Woude.** 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science **280:**734–737.
12. **During, R. L., W. Li, B. Hao, J. M. Koenig, D. S. Stephens, C. P. Quinn, and F. S. Southwick.** 2005. Anthrax lethal toxin paralyzes neutrophil actin-based motility. J. Infect. Dis. **192:**837–845.
13. **Friedlander, A. M.** 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. **261:**7123–7126.
14. **Friedlander, A. M., R. Bhatnagar, S. H. Leppla, L. Johnson, and Y. Singh.** 1993. Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. Infect. Immun. **61:**245–252.
15. **Gadebusch, H. H.** 1960. Specific degradation of *Cryptococcus neoformans* 3723 capsular polysaccharide by a microbial enzyme. II. Biological activity of the enzyme. J. Infect. Dis. **107:**402–405.
16. **Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins.** 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect. Immun. **49:**291–297.
17. **Hu, H., Q. Sa, T. M. Koehler, A. I. Aronson, and D. Zhou.** 2006. Inactivation of *Bacillus anthracis* spores in murine primary macrophages. Cell. Microbiol. **8:**1634–1642.
18. **Ivins, B. E., J. W. Ezzell, Jr., J. Jemski, K. W. Hedlund, J. D. Ristroph, and S. H. Leppla.** 1986. Immunization studies with attenuated strains of *Bacillus anthracis*. Infect. Immun. **52:**454–458.
19. **Joyce, J., J. Cook, D. Chabot, R. Hepler, W. Shoop, Q. Xu, T. Stambaugh, M. Aste-Amezaga, S. Wang, L. Indrawati, M. Bruner, A. Friedlander, P. Keller, and M. Caulfield.** 2006. Immunogenicity and protective efficacy of *Bacillus anthracis* poly-gamma-D-glutamic acid capsule covalently coupled to a protein carrier using a novel triazine-based conjugation strategy. J. Biol. Chem. **281:**4831–4843.
20. **Kang, T. J., M. J. Fenton, M. A. Weiner, S. Hibbs, S. Basu, L. Baillie, and A. S. Cross.** 2005. Murine macrophages kill the vegetative form of *Bacillus anthracis*. Infect. Immun. **73:**7495–7501.
21. **Keppie, J., P. W. Harris-Smith, and H. Smith.** 1963. The chemical basis of the virulence of *Bacillus anthracis*. IX. Its aggressins and their mode of action. Br. J. Exp. Pathol. **44:**446–453.
22. **Keppie, J., H. Smith, and P. W. Harris-Smith.** 1953. The chemical basis of the virulence of *Bacillus anthracis*. II. Some biological properties of bacterial products. Br. J. Exp. Pathol. **34:**486–496.
23. **Kozel, T. R., W. J. Murphy, S. Brandt, B. R. Blazar, J. A. Lovchik, P. Thorkildson, A. Percival, and C. R. Lyons.** 2004. mAbs to *Bacillus anthracis* capsular antigen for immunoprotection in anthrax and detection of antigenemia. Proc. Natl. Acad. Sci. USA **101:**5042–5047.
24. **Kozel, T. R., P. Thorkildson, S. Brandt, W. H. Welch, J. A. Lovchik, D. P. Aucoin, J. Vilai, and C. R. Lyons.** 2007. Protective and immunochemical activities of monoclonal antibodies reactive with the *Bacillus anthracis* polypeptide capsule. Infect. Immun. **75:**152–163.
25. **Leppla, S. H.** 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc. Natl. Acad. Sci. USA **79:**3162–3166.
26. **Makino, S., I. Uchida, N. Terakado, C. Sasakawa, and M. Yoshikawa.** 1989. Molecular characterization and protein analysis of the cap region, which is essential for encapsulation in *Bacillus anthracis*. J. Bacteriol. **171:**722–730.
27. **Makino, S., M. Watarai, H. I. Cheun, T. Shirahata, and I. Uchida.** 2002. Effect of the lower molecular capsule released from the cell surface of *Bacillus anthracis* on the pathogenesis of anthrax. J. Infect. Dis. **186:**227–233.
28. **Mayer-Scholl, A., R. Hurwitz, V. Brinkmann, M. Schmid, P. Jungblut, Y. Weinrauch, and A. Zychlinsky.** 2005. Human neutrophils kill *Bacillus anthracis*. PLoS Pathog. **1:**179–186.
29. **Montville, T. J., T. De Siano, A. Nock, S. Padhi, and D. Wade.** 2006. Inhibition of *Bacillus anthracis* and potential surrogate bacilli growth from spore inocula by nisin and other antimicrobial peptides. J. Food Prot. **69:**2529–2533.
30. **Mushtaq, N., M. B. Redpath, J. P. Luzio, and P. W. Taylor.** 2004. Prevention and cure of systemic *Escherichia coli* K1 infection by modification of the bacterial phenotype. Antimicrob. Agents Chemother. **48:**1503–1508.
31. **Mushtaq, N., M. B. Redpath, J. P. Luzio, and P. W. Taylor.** 2005. Treatment of experimental *Escherichia coli* infection with recombinant bacteriophage-derived capsule depolymerase. J. Antimicrob. Chemother. **24:**160–165.

31a. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.

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32. **O'Brien, D. K., W. J. Ribot, S. A. Tobery, and A. M. Friedlander.** 2005. Bacillus ACT 2005, abstr. P97.
33. **O'Brien, D. K., A. Scorpio, D. J. Chabot, R. I. Lehrer, and A. M. Friedlander.** 2005. Bacillus ACT 2005, abstr. P96.
34. **O'Brien, J., A. Friedlander, T. Dreier, J. Ezzell, and S. Leppla.** 1985. Effects of anthrax toxin components on human neutrophils. Infect. Immun. **47:**306–310.
35. **Piris-Gimenez, A., M. Paya, G. Lambeau, M. Chignard, M. Mock, L. Touqui, and P. L. Goossens.** 2005. In vivo protective role of human group IIa phospholipase A2 against experimental anthrax. J. Immunol. **175:**6786–6791.
36. **Radyuk, S. N., P. A. Mericko, T. G. Popova, E. Grene, and K. Alibek.** 2003. In vitro-generated respiratory mucosa: a new tool to study inhalational anthrax. Biochem. Biophys. Res. Commun. **305:**624–632.
37. **Schneerson, R., J. Kubler-Kielb, T. Y. Liu, Z. D. Dai, S. H. Leppla, A. Yergey, P. Backlund, J. Shiloach, F. Majadly, and J. B. Robbins.** 2003. Poly(gamma-D-glutamic acid) protein conjugates induce IgG antibodies in mice to the capsule of *Bacillus anthracis*: a potential addition to the anthrax vaccine. Proc. Natl. Acad. Sci. USA **100:**8945–8950.
38. **Schuch, R., D. Nelson, and V. A. Fischetti.** 2002. A bacteriolytic agent that detects and kills *Bacillus anthracis*. Nature **418:**884–889.
39. **Scorpio, A., D. J. Chabot, W. A. Day, D. K. O'Brien, N. J. Vietri, Y. Itoh, M. Mohamadzadeh, and A. M. Friedlander.** 2007. Poly-γ-glutamate capsule-degrading enzyme treatment enhances phagocytosis and killing of encapsulated *Bacillus anthracis*. Antimicrob. Agents Chemother. **51:**215–222.
40. **Wang, T. T., P. F. Fellows, T. J. Leighton, and A. H. Lucas.** 2004. Induction of opsonic antibodies to the gamma-D-glutamic acid capsule of *Bacillus anthracis* by immunization with a synthetic peptide-carrier protein conjugate. FEMS Immunol. Med. Microbiol. **40:**231–237.
41. **Wang, W., C. Mulakala, S. C. Ward, G. Jung, H. Luong, D. Pham, A. J. Waring, Y. Kaznessis, W. Lu, K. A. Bradley, and R. I. Lehrer.** 2006. Retrocyclins kill bacilli and germinating spores of *Bacillus anthracis* and inactivate anthrax lethal toxin. J. Biol. Chem. **281:**32755–32764.
42. **Welkos, S. L., R. W. Trotter, D. M. Becker, and G. O. Nelson.** 1989. Resistance to the Sterne strain of *B. anthracis*: phagocytic cell responses of resistant and susceptible mice. Microb. Pathog. **7:**15–35.
43. **Wright, G. G., and G. L. Mandell.** 1986. Anthrax toxin blocks priming of neutrophils by lipopolysaccharide and by muramyl dipeptide. J. Exp. Med. **164:**1700–1709.

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